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(74) Agent: **STRAUS, Alexander**; Becker, Kurig, Straus, Bavariastrasse 7, D-80336 München (DE).

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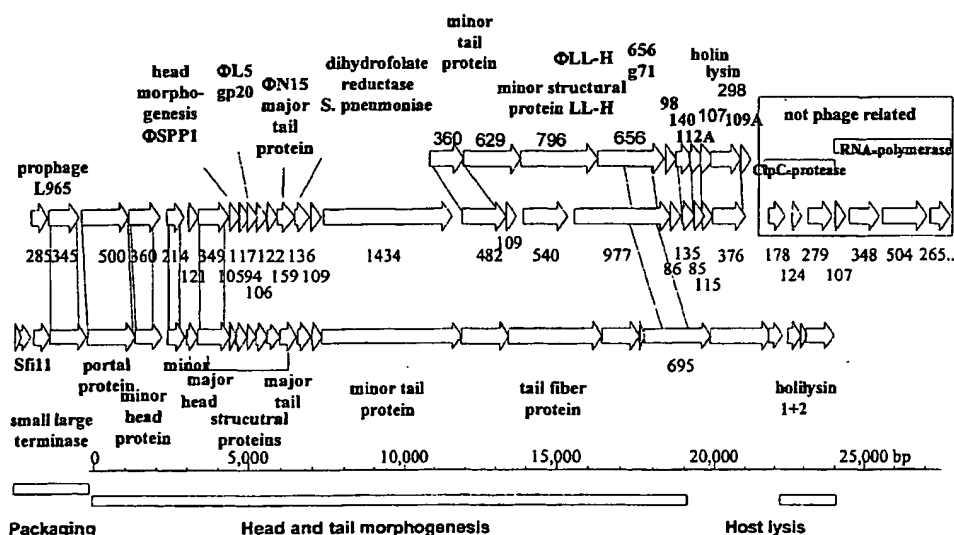
(71) Applicant (*for all designated States except US*): **SOCIETE DES PRODUITS NESTLE S.A.** [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **BRUESSOW, Harald** [DE/CH]; Chemin de la Chaumény 13, CH-1814 La Tour de Peilz (CH). **DESIERE, Frank** [BE/CH]; Avenue

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(54) Title: PROPHAGE DNA SEQUENCES AND THEIR USE IN DETERMINING INSTABILITY IN LACTOBACILLI CULTURES

Alignment of the gene map of L965 and the partial gene map of *Streptococcus thermophilus* bacteriophage S611 and *Lactobacillus delbrueckii* bacteriophage LL-H.(57) Abstract: The present invention relates to prophage DNA sequences of *Lactobacillus johnsonii*, in particular to prophages lysin and or holin genes in *Lactobacillus johnsonii* and their use in determining and/or preventing the instability of *Lactobacilli* cultures.

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**Prophage DNA sequences and their use in determining instability
in lactobacilli cultures**

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The present invention relates to prophage DNA sequences of *lactobacillus johnsonii*, in particular to prophage lysin and or holin genes in *lactobacillus johnsonii* and their use in determining and/or extinguishing instability of lactobacilli cultures.

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Lactobacilli are used in factories for the production of dairy products such as yogurt, milk drinks, and white cheese. Since the introduction of such strains in applications on industrial scale no substantial phage problems have been reported. Yet, due to the increasing number of applications more and more fermentation failures were notified, which could not be attributed to bacterial contamination.

15

The problems reported were not observed in initial small fermenter cultures, yet during fermentation on large scale the number of lactobacilli reached a level that constantly dropped during further fermentation to up to 80 to 90 % of the initial cell count. This drop of the viable cell number could not be explained by lytic phage infection which causes no measurable problems at low phage titers but produces a dramatic, greater than 90% loss at high phage titers.

20

It was initially hypothesized that these problems might be merely due to nutritional conditions prevailing in large scale fermenters, such as e.g. a lack of growth factors in the larger volumes, or due to technical circumstances, e.g. linked to the mechanical treatment of the cells. Also genetic inclinations were envisaged wherein gene expression of the cells were deemed to change under conditions of mass culture wherein a bacterial suicide system could be induced (Lepeuple et al., Appl. Environ. Microbiol. 64 (1998), 4142-4148).

25

Consequently a problem of the present invention is to find means for determining and/or extinguishing biological factors responsible for instabilities in factory fermentations.

5 The above problem has been solved by providing the nucleotide sequence of the prophages contained in *Lactobacillus johnsonii* La1 CNCM I 1225, identified by SEQ ID No. 1, 2 and 3.

These sequences or parts thereof may suitably be used for determining the presence of prophages in *Lactobacilli*.

10 According to another preferred embodiment the above sequences or parts thereof may be used for extinguishing active prophages from *lactobacilli* genomes so as to improve the stability of *lactobacilli* cultures.

15 Fig. 1, 2 and 3 show the prediction of open reading frames attributed to phage sequences and their respective polypeptides

Prophages are the genomes of temperate phages that have integrated into the bacterial genome encoding inter alia lytic enzymes to free the phage from the cell after induction. Autolysins are one group of such lytic enzymes, polypeptides that en-zy-ma--tically act on the bacterial cell wall. The liberation of some prophages from the genome may be induced by mutagens, such as DNA damaging agents (e.g. mitomycin C) or UV light and are also activated when the cell experiences other stress situations. Though prophage DNA present in the bacterial genome may be incomplete and is hence in-ca--pable of producing viable phage particles they may still be capable of encoding lytic enzymes.

25 During the experiments leading to the present invention it was therefore decided to investigate the genome of *L. johnsonii* for the presence of prophages, in order to exclude the possibility that temperate phages may be responsible for the instability of *latobacilli* cultures. This screening was not trivial, since no genetic information on the structure of *L. johnsonii* phages was known.

The La1 genome dataset available from the Genome project was screened with bacterio-phage sequences of known Lactobacillus, Lactococcus, Streptococcus and Bacillus phages using the BLAST algorithm. As first screening probes the following phage sequences were used:

- 5 • Bacteriophage LL-H, accession number M96254
 (Vasala et al., Appl. Environ. Microbiol 61 (1995), 4004 - 4011)
- Bacteriophage SPP1 complete nucleotide sequence, X97918
 (Becker et al., J. Mol. Biol. 268 (1997), 822-839)
- B. subtilis DNA (28 kb PBSX/skin element region), Z70177
10 (Krogh et al., Microbiology. 142 (1996), 2031-2040)
- Lactobacillus bacteriophage phig1e complete genomic DNA, X98106
 (Kodaira et al., Gene 187 (1997), 45-53)

When the La1 genome database was searched with the set of phage sequences described three
15 different contigs (the La1 DNA dataset is organized in segments termed "contigs", which range
in size from about 500 bp to about 50 kb) revealed phage-related sequences. These contigs were
retrieved from the database and then further analysed. In these 3 contigs of various lengths,
different amounts of bacteriophage related sequences were found.

20 In the contig termed L965 (about 35 kb total length) (SEQ ID No. 1), a 25 kb segment of
contiguous phage sequences was identified, which codes for a complete packaging,
morphogenesis and lysis module of a pac-site containing temperate Siphoviridae most closely
related to S. thermophilus phage Sfi11.

25 The contig L771 (about 16 kb) (SEQ ID No. 2) contains 8kb of phage related sequences, most
closely related to B. subtilis prophage PBSX. In contig L771 also a probable lysis module could
be identified.

The contig L928 (11kb) (SEQ ID No. 3) codes for a contiguous phage sequence, which spans
30 from the replication region to the packaging region. The contigs are discussed in detail in the
following paragraphs.

Prophage contig L965

Contig L965 is 35.6 kb long and contains 32 open reading frames. Database homology searches revealed 15 highly significant matches and 3 matches of borderline significance. The results of these searches are summarized in table 1.

Table 1

L965 open reading frames and database similarities. The grey bar indicates the end of phage-related sequences. ORF's are named by the number of aminoacids in the predicted protein, the BLAST P-value and the scores are given, identity is on aa level.

ORF	Similarity	BLAST	Identity
89	-		
285	XTMA_BACSU PBSX PHAGE TERMINASE SMALL SUB. Hypoth. protein 8 (xre region) Bacillus subt YQAS_BACSU similar to phage-related terminase small subunit Bacillus subtilis (Z99110) similar to hypoth. Proteins from.	7e-16 5e-14 2e-08 2e-08	62/229 (27%) 48/150 (32%) 27/49 (55%) 27/49 (55%)
345	(U88974) ORF26 S. thermophilus phage 01205 (AE000790) cons. Hyp. Protein Borrelia (U19754) BBA31 homolog Borrelia burgdorferi	5e-16 1e-06 4e-06	72/251 (28%) 45/212 (21%) 66/306 (21%)
500	(AF057033) gp502 S. thermophilus phage Sfi11 (U88974) ORF27 S. thermophilus phage 01205 (Y11901) Lactococcus lactis SIZ_BPSP portal portal vertex protein SPP1	2e-39 2e-38 4e-16 1e-08	127/446 (28%) 123/446 (27%) 70/259 (27%) 106/480 (22%)
360	gp284 S. thermophilus pahge Sfi11 ORF28 S. thermophilus phage 01205	2e-09 5e-09	39/155 (25%) 75/341 (21%)
214	(U88974) ORF2 S. thermophilus phage 01205 (AF057033) gp193 S. thermophilus bacteriophage Sfi11	5e-10 2e-09	49/169 (28%) 48/168 (28%)
121	(AF071201) bacteriophage Felix 01	3.4	16/53 (30%)
349	(AF057033) gp348 S.thermophilus phage Sfi11 (U88974) ORF31 S. thermophilus phage 01205	6e-24 1e-23	88/311 (28%) 87/311 (27%)
105	(Q38584) product required for head morphogenesis (AF009630) E14 bacteriophage bIL170 (O21901) bacteriophage sk1	0.00014 0.0087 0.011	24/98 (24%) 11/61 (18%) 11/61 (18%)
117		No	Similarity
94	Mycobacteriophage L5 gp20	0.30	

106 122		No	similarity
159	INVA_YEREN Invasin Yersinia enterocolitica (O64327) gp13 Bacteriophage N15	3e-05 0.0032	32/153 (20%) 32/237 (23%)
136	(AF055723) Dihydrofolate reductase Streptococcus pneumoniae	5e-05	33/123 (26%)
109			
1434	Myosin		
482	(AF032121) S. thermophilus phage Sfi21 orf 1560 (AF032122) S. thermophilus phage Sfi19 orf 1620 (L02496) phage LL-H orf 999 (X98106) minor capsid protein phage phigle orf1608 YQBO_BACSU XKDO_BACSU PBSX protein XKDO (AB009866) orf 16 bacteriophage phi PVL (AB009866) orf 17 phage phi PVL (AF009630) 116 bacteriophage bIL170	161 1e- 38 150 2e- 35 147 1e- 34 102 4e- 21 86 5e-16 83 4e-15 53 3e-06 40 0.026 32 5.7	92/216 (42%) 99/262 (37%) 102/273 (37%) 64/167 (38%) 84/303 (27%) 77/244 (31%) 0/235 (25%) 32/114 (28%) 25/96 (26%)
109	?		
540	(AF053947) lambda host specific protein J Yersinia pestis YHYA_BPH44 hyp. 65 kd prot. Hyaluronidase B30566 S. pyogenes phage H4489A (M19348)ORF S. pyogenes phage H4489A	43 0.003 43 0.003	28/135 (20%) 28/132 (21%)
977	(L02496) minor structural protein gp58 LL-H (U42580) Paramecium bursaria Chlorella virus (AF032121) S. thermophilus phage Sfi21 orf1276 (X97918) gene 21 Bacteriophage SPP1 (L48605) minor structural protein Lactococcus phage c2	58 2e-07 51 4e-05 39 0.11 41 0.036 37 0.31	38/118 (32%) 160/789 (20%) 16/44 (36%) 65/245 (26%) 17/38 (44%)
86	-		
135	(L02496) orf140 Bacteriophage LL-H	52 2e-06	32/100 (32%)
115	(L02496) holin Bacteriophage LL-H	36 0.052	27/99 (27%)
376	(X78410) lysin Lactobacillus bacteriophage phi adh (L02496) muramidase Bacteriophage LL-H (Z26590) LysA Bacteriophage mv4 LYCA_BPMV1 endolysin mv1 lysin	367 e- 101 224 5e- 58 214 8e- 55 137 1e- 31	184/307 (59%) 102/222 (45%) 70/179 (39%) 70/179 (39%)
178	CLAB_LYCES ATP-dependent clp protease CLPC PORPU, CLPA PEA, CLAA LYCES,	95 2e-19 95 3e-19	
124	(U40604) ClpC ATPase Listeria monocytogenes MECB_BACSU neg. reg. Of gen. Comp. ClpC B. subtilis CLAA_LYCES clp protease ATP-binding sub.	177 2e- 44 175 7e- 44 169 5e- 42	

279	"	"	
107	"	"	
348	RPOB_BACSU DNA-directed RNA polymerase RPOB_STAAU DNA-directed RNA polymerase	448 e- 125 426 e- 118	
504	"	"	
265	"	"	

BLAST scores and identities in *italics* are derived by a PAM 250 "substitution matrix", which assigns a score for aligning any possible pair of residues. The default substitution matrix in BLAST is usually BLOSUM62

16 matches were found with known proteins from bacteriophages. These matches were clustered in the left 80% of the contig. The similarities were found to phages from *Streptococcus*, *Bacillus* and *Lactobacillus*. Five predicted adjacent ORF's (open reading frame) showed similarity with *S. thermophilus* phage fSfi11 and 01205. Two further non-adjacent genes showed similarity with *S. thermophilus* phages fSfi11 and fSfi21, respectively (Fig. 1). Further, four adjacent genes showed similarity with genes from *L. delbrueckii* subsp. *lactis* bacteriophage fLL-H and *Lactobacillus gasseri* fadh, respectively. One gene shows significant similarity to the cryptic *Bacillus subtilis* prophage PBSX. Three genes located in the right 20% of the contig showed significant similarity to bacterial proteins.

Six contiguous genes from *Johnsonii* La-1 showed not only a similar topological arrangement to corresponding genes from *S. thermophilus* fSfi11 (Fig. 1), but also (except for one predicted protein) highly significant sequence similarities at the amino acid (aa) level. This observation indicates a close evolutionary relationship between phages infecting evolutionary related bacterial hosts as has previously been deduced from the comparative analysis of other genome segments from *S. thermophilus* phages (tail module, lysogeny modules).

Over the next ten genes a very similar topological arrangement was also seen between both phages: A very characteristic pattern of 8 relatively small genes followed by a very large gene was found in both the *L. johnsonii* La-1 genome sequence and in the Sfi11 phage sequences, with a striking similarity in ORF length (Fig. 1). However, no sequence similarity was observed

between both groups of genes. Previously a similar genomic organization in phage lambda and phages from low GC gram-positive bacteria was observed (Chandry et al., Mol. Microbiol. 26, (1997) 49-64). This parallel allowed the tentative prediction of gene functions based on detailed knowledge of phage lambda. Several of these predictions have been verified in the analysis of the

5 *S. thermo-philus* phage genomes. According to this new phage bioinformatic concept which is based on the hypothesis of distant evolutionary relationships between Siphoviridae (tailed phages) from many bacterial genera, the following functions for the newly described *L. johnsonii* La-1 ORF's could be predicted (Table 1). In four cases the tentative attribution of function was supported by bioinformatic links when using a sensitive PAM 250 scoring matrix (see below).

10 According to the lambda model, one would predict ORF 159 to encode the major tail protein of prophage L965 from *L. johnsonii* La-1.

Table 2

Prediction of gene functions for the La-1 ORF's
by comparison with phage lambda gene map

La-1 ORF	Lambda gene	Lambda ORF	Function	BI link
285	Nu1	181	Terminase small subunit	yes
345	A	641	Terminase large subunit	
?	W		Head-tail joining	
500	B	533	Portal protein	yes
360	C	439	Capsid component	yes
214	Nu3	201	Scaffold protein	yes
121	D	110	Head-DNA stabilisation	
349	E	341	Major head protein	yes
105	W	68	Head-tail joining	(yes)
117	FI	117	Terminase-vertex binding	
94	FII	117	Head-tail joining	
106	Z	192	Links DNA ends to tail	
122	U	131	Head-tail joining	
159	V	256	Major tail protein	(yes)
136	G	140	Initiator formation, injection	
109	T	144	Tail component	
1434	H	853	Tail ruler, injection	(yes)

The first column gives the length of the La-1 prophage genes in aa. The genes are noted as they follow on the genetic map.

The second column gives the corresponding lambda genes when the two phage genomes were aligned. For optimal alignment the W gene is quoted in the topological order found in lambdoid phage P21.

The third column gives the aa length of the corresponding lambda genes.

The fourth column gives the biological function of the corresponding lambda proteins.

The fifth column indicates whether a bioinformatic link from database searches confirmed the tentative gene attribution.

5

Taken together, the leftmost 17 kb of contig L965 comprising 18 ORF's (ORF 285 to ORF 482) showed clear topological similarity to the genomic organisation of a number of Siphoviridae from various bacterial hosts. Sequence similarity over this genome segment was mainly with predicted proteins from *S. thermophilus* phages. An interesting aspect was the link with proteins from both cos-site and pac-site *S. thermophilus* phages. The head genes of L965 prophage showed similarity to pac-site of *S. thermophilus* phages such as fSfi11 or fO1205 while a tail gene (ORF 482) showed sequence similarity with cos-site phages (table 1).

In *S. thermophilus* phages the large minor tail protein gene is followed by four (cos-site phages) to six genes (pac-site phages) before reaching the lysis cassette. In *L. johnsonii* La-1 prophage L965 six genes were found between the analogue of the minor tail protein and the lysis cassette.

ORF 115 gp showed 27 % aa identity to the holin from *L. delbrueckii* phage LL-H. Both proteins showed a almost identical predicted transmembrane profile.

20

ORF 376 gp showed 59% aa identity with the lysin from *Lactobacillus gasseri* phage adh over its entire length (P=10-101) and 45 % with *L. delbrueckii* phages LL-H over the N-terminal 220 aa (P=10-58). It should be noted that the lysin of LL-H is about 80 aa shorter than the L965 lysin. Significant similarity with numerous other phage lysins (*Lb. delbrueckii* phages mv4, mv1; *S. pneumoniae* phages CP-1; CP-7, CP-9; *L. lactis* phages Tuc2009, LC-3; TP21; all P values < 10-8) and an autolytic lysozyme from *Clostridium acetobutylicum* (ORF 324; P=10-5) were detected.

Notably, the ORF 977 to ORF 376 region of prophage L965 was similarly organised as the lysis module and its preceding genes in *L. delbrueckii* phage LL-H (Fig. 1). In addition to the similar topological organisation of the ORF's, three non-adjacent genes predicted proteins with sequence similarity to the corresponding LL-H proteins.

30

The L965 prophage encodes a holin and a lysin protein which resemble closely proteins from Lactobacillus phages. The phage lysins normally lack secretion signals. This was also the case in the lysins predicted for the two La-1 prophages. The phage lysins are thus unable to reach the target of their enzymatic action: the cell wall of the gram-positive bacterium. Export of the phage lysin is mediated by holins (Krogh et al., J. Bacteriol. 180 (1998), 2110-2117). The holins are pore-forming proteins which allow the export of intracellular proteins. There are different forms of holins.

- 10 Class I holins could be identified, which span the cytoplasmic membrane three times. Expression of holins is lethal to the cell, even in the absence of lysin expression.

ORF 376 was followed by a relatively long non-coding region of about 800 bp and then by two genes of bacterial host origin encoding ClpA (P=10-19) and ClpC proteins (P=10-44) of the clp protease system, two unattributed genes and a gene encoding a DNA-directed RNA polymerase (P=10-125).

Prophage contig L771

- 20 Contig L771 consists of 15.5 kb of sequence information. A tentative gene map (Fig. 2) was established and phage related sequences were found in the left part of contig L771 (0-7 kb), while from 7 kb onwards unequivocal bacterial genes were identified (ORF 131 gp: PemK, P=10-9; ORF 206 gp: peptide methionine sulfoxide reductase P=10-51). The first part showed topologically a very similar organisation to the late gene region of the B. subtilis prophage PBSX. This region comprises a number of unattributed genes (xkdT to xkdX) followed by a lysis cassette. The similarity was not limited to the topological arrangement of the genes, as several genes from the L771 prophage sequence predicted also proteins which showed sequence similarity with the corresponding PBSX prophage proteins.
- 25
- 30 Clear bioinformatic links were established for the genes in this region. The matches allowed the association of these genes with the corresponding PBSX genes. ORF 231 gp showed 37 %

identity over a 60 aa segment of an autolytic lysozyme of *Clostridium acetobutylicum* ($P=10^{-4}$). ORF 227 gp showed over a 75 aa segment high similarity to lysins of the amidase class from a number of bacteriophages (*Lactobacillus* phage adh, g1e; Tp-21; all P values $< 10^{-10}$). The highest similarity was with phage adh (75% identity, $P=10^{-31}$). This highly conserved phage
5 lysin segment was also found in the putative lysin from prophage L965 of *L. johnsonii* La-1, notably a 330 bp segment of the lysin genes from both *L. johnsonii* La-1 prophages shared 70 % bp identity.

According to their position upstream of lysin genes ORF 93 and ORF 92 clearly identify
10 themselves as holin genes. Two arguments are in favour of a holin attribution to ORF 93: class I holin genes encode proteins of about 100 aa length and these proteins show three transmembrane segments.

Contig L928

15 Contig L928 contains 11 kb of sequence data. The sequence predicts 19 proteins (Fig. 3) which show similarities to *S. thermophilus*, *B. subtilis* and *Lactobacillus* bacterio-phages.

The right 7 kb show some similarity to the topological organization of the pac-site region of *S.*
20 *thermophilus* phage Sfi11, but the sequence similarity was only significant for the putative portal protein. In addition the potential small and large subunits of the terminase were identified.

The sequence information provided may now be utilized for determining the presence of related sequences in lactobacilli, also other than lactobacilli *johnsonii*. To this end, a part of the
25 sequence of one of the contigs disclosed, preferably sequences comprising ORF's for holins or autolysins, may be used as probes in hybridisation or experiments using various conditions of stringency. Moreover, the technique of polymerase chain reaction may well be used for this purpose.

30 Likewise, the sequence information may be used for extinguishing such sequences detrimental to the growth of lactobacilli. In particular, the genes coding for autolysins and/or holins,

respectively, may be deactivated or deleted from the genome of the lactobacillus of interest making use of homologous recombination. Hence flanking regions of each of the contigs or of the respective genes to be deleted/inactivated may be used for effecting homologous recombination, by introducing a DNA sequence containing a deactivated gene or a marker gene in between the respective "flanking sequences" into the bacteria's genome. The DNA segment will then insert at the location of homologous sequences in the chromosome and delete the endogeneous sequences. To this end the use of a marker gene may be suitable to select clones, in which the endogeneous contig's or DNA-sequences have been replaced by the recombinant one.

Further the sequences or part thereof, in particular short oligonucleotides may be used for monitoring the presence or loss of any prophages in Lactic acid bacteria by DNA/DNA hybridization or PCR reaction. Likewise, the DNA sequences provided by the present invention or parts thereof, such as oligonucleotides of desired length, may be used for monitoring prophage gene expression under different growth conditions, such that the optimal fermentation and growth conditions for the lactic acid bacteria may be selected.

The following examples illustrate the invention without limiting it thereto.

Examples

Fragment cloning and clone-bank construction

One mg samples of *L. johnsonii* Lal chromosomal DNA was digested by 6 base pair sequence recognition restriction enzymes in the search of digestions producing predominantly large, i.e. 8 kb and larger DNA fragments. Seven restriction enzymes identified in this way, BamHI, SacI, SphI, BsrGI, NheI, KpnI and MluI were selected for cloning. 0.3 mg of chromosomal DNA was digested to completion with each of the 7 restriction enzymes and ligated with 0.1 mg of the appropriately prepared pUC19 (Yanisch-Perron et al., Gene 33 (1985), 103-119) (contains a unique MluI site, RDP unpublished results). These ligations were electro-transformed into the *E. coli* strain BZ234 and directly plated onto LB plates supplemented with 100 mg/ml ampicillin (Boehringer Mannheim, product number 835 242), Xgal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, Boehringer Mannheim, product number 1 680 293) and IPTG (isopropyl-b-

D-thio-galacto-side, Boehringer Mannheim, product number 1 411 446). The plates were incubated at 37°C for 16 h and the insert containing colonies were identified by the blue-white color reaction, i.e. white colonies containing inserts. White colonies were picked into 96-well microtiter plates containing 150 ml LB medium supplemented with 100 mg/ml ampicillin and
5 incubated at 37°C to produce mini cultures.

From these microtiter plates, 20 ml aliquots were removed from each of the 12 wells in a given row and pooled in 8 individual Eppendorf tubes to give the 'pools of rows' (i.e. A1-12 etc). From these 'pools of rows', 40 ml aliquots were taken into fresh Eppendorf tubes to give the 'pools of
10 plates'. Finally, glycerol was added to the microtiter plates plus the pools in the Eppendorf tubes and after mixing, frozen at -20°C.

Eight microtiter plates were inoculated with colonies from each of the 7 ligations giving more than 5000 individual clones.

15

Clone-bank testing

The quality of the clone-banks in the microtiter plates was tested by restriction analysis of isolated plasmids to estimate the size of the inserted *L. johnsonii* La1 fragments and the level of repeated fragments. Thirty ml of the microtiter culture were inoculated into 3 ml of LB medium
20 supplemented with 100 mg/ml ampicillin and incubated at 37°C with agitation. Small scale plasmid isolations were prepared using the Genomed mini prep kit (product number 200200) as described in the instructions leaflet and finally suspended in 50 ml TE buffer. Up to 100 plasmids from each of the 7 clone-banks were prepared, digested with the restriction enzymes
25 *EcoRI* plus *HindIII* and the fragments resolved on a 1% agarose gel. These restriction enzymes cut at the two extremities of the cloning arrays of the pUC plasmids and thus release the vector from the La1 cloned insert that may be further digested if these sites are present within the insert DNA. This produces a plasmid digestion pattern, or a sort of code-bar pattern, that is specific in the number and sizes of the DNA fragments cloned from *L. johnsonii* La1. The direct
30 comparison of these patterns can rapidly eliminate clones that are represented more than once in the clone-bank, regardless of the orientation of the inserted DNA. The sizes of the La1 *EcoRI* plus *HindIII* restriction fragments may also be summed to produce the total size of that La1

insert. From these data we have a conservative estimate for an average 4-5 kb insert size, with few clones below this size and with many clones reaching 10 kb and larger. We estimate that each of the 7 clone-banks contains approximately 2 times the *Lal* genome complement.

- 5 The plasmids also showed an impressive physical stability and a high variation in copy number that depended on the nature of the cloned insert. While pUC19 and pUC21 are capable of replication at up to 750 copies/bacterium (and most clones showed this high copy number) some clones displayed a greatly reduced copy number that reflected an *E. coli* related toxicity of the cloned DNA. These reduced copy number plasmids varied to a point where they were no longer
10 visible on the *EcoRI* plus *HindIII* test digestions, with a copy number estimated at 50 copies/bacterium or less.

DNA sequencing

- Sequencing of the *L. johnsonii* *Lal* cloned inserts was routinely achieved using the Thermo
15 Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, product number RPN 2538) and the IRD-800 labelled pUC forward and reverse primers. Primer/template mix contained 4 ml of standard template and 4ml primer at 1 pMol/ml in 25 ml volume. Add 6 ml primer/template mix to each of 4 tubes of 200 ml capacity in strip form, plus 2 ml of the sequencing mix, i.e. A to the A reaction etc. The tubes were sealed, mixed and the
20 sequencing took place in an Omni thermocycler using the following conditions; 5 min at 95°C, followed by 25 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min and finally held at room temperature. Three ml of stop solution were added and the samples were frozen at -20°C.

- A sample volume of 0.8 ml was loaded onto the Licor DNA sequencer model L4000 with 66 cm
25 plates and 0.25 mm gel thickness. Electrophoresis and sequence reading was by the Licor sequencer. Sequences were downloaded to the GCG suite of programs for sequence compilation and analysis.

PCR screening of clone banks

- 30 The structure of the 7 clone-banks with their resulting pools was designed to facilitate the rapid screening of the clones by PCR. A known DNA sequence taken from the end of a sequenced

clone was used to design a pair of PCR primers to produce a PCR amplification product of between 400 and 600 base pairs. A 1 ml sample from the 'pools of plates' was mixed with the PCR primers in a 50 ml volume in the 8 x 200 ml strips with dNTPs, enzyme buffer and 1.25 units of SuperTaq. The reaction volume was heated to 95°C for 5 min in a Perkin-Elmer 9700 PCR machine, then amplified for 30 cycles of 95°C 1 for min, 40°C 1 for min, 72°C for 2 min and finally held at 4°C. 10 ml of gel loading dye was added, mixed and loaded onto a 1.5% agarose gel and electrophoresed at 100 mA for 1 - 2 hrs. A positive control with 200 ng of *L. johnsonii* La1 chromosomal DNA also used for a PCR reaction and as a size marker for the amplification product and a control of the PCR reaction. The presence of a single positive clone in the mixture of 96 clones in the microtiter plate may be directly seen by the presence of a PCR product the same size as that of *L. johnsonii* La1. This technique allows the rapid screening of all the clone-banks in less than 5 hr without the use of radioactivity. Once a positive clone is identified, the PCR detection is repeated on the 8 "pools of rows" for that microtiter plate, and finally on the 12 wells in the identified row.

In case the plasmids are of too low a copy number to be sequenced directly, the plasmids are used as templates for long-range PCR with the appropriate primer from the detection reaction and either oligonucleotide 6617 (5'ACGCCAGCTGGCGAAAGGGGG3') (SEQ ID No. 4) or 6618 (5'GCTCACTCATTAGGCACCCCAGGC3') (SEQ ID No. 5) which amplifies from the pUC19 vector towards the insert. The PCR product was then gel purified, the DNA eluted and sequenced directly as described for the plasmids.

Sequence analysis

The Genetics Computer Group sequence analysis package (University of Wisconsin) was used to analyze the sequences. Nucleotide (nt) and predicted amino acid (aa) sequences were compared to those in the databases (GenBank, Release 105; EMBL (Abridged), Release 53; PIR-Protein, Release 55; SWISS-PROT, Release 35; PROSITE, Release 14.0) using FastA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) programs.

Open reading frames (ORF) have been predicted using Clonemanager version 5.0 using ATG and GTG as possible start codons and a minimum size of 90 aa.

Mitomycin C induction test

The test was adapted from Arber et al., Lambda II (Hendrix, R.W., Ed.) Cold Spring Harbour Laboratory (1983), 443-445. 0.1 ml of a fresh culture was inoculated into a tube containing 10 ml
5 M17 medium supplemented with 0.5 ml of lactose (10% w/v). The culture was subsequently incubated for 1 h at 40° C. Mitomycin C was then added to the final concentration of 0.1 µg per ml. After 6 hours of further incubation the culture was examined by testing the optical density and a plaque assay.

10 PCR

The culture supernatant was treated with DNase to destroy any contaminating, naked chromosomal DNA from lysed bacteria prior to the extraction of the phage DNA (the phage DNA is protected by the phage proteins). PCR was done with primers specific for the L965 prophage.

Claims

- 5 1. DNA sequence identified by SEQ ID No. 1
2. DNA sequence identified by SEQ ID No. 2
3. DNA sequence identified by SEQ ID No. 3
- 10 4. Use of a DNA-sequence according to any of the preceding claims or parts thereof for determining the presence of prophages in lactic acid bacteria.
5. Use of a DNA-sequence according to any of the claims 1 to 3 or parts thereof for
15 extinguishing active DNA sequences responsible for killing of lactic acid bacteria.
6. The use according to claim 5, wherein the active DNA sequences encode holins.
7. The use according to claim 5, wherein the active DNA sequences encode lysins.
- 20 8. The use of a DNA sequence according to any of claims 1 to 3 or parts thereof to monitor the presence or loss of any prophages in lactic acid bacteria, preferably lactobacilli.
- 25 9. The use of a DNA sequence according to any of claims 1 to 3 or parts thereof to monitor prophage gene-expression in lactic acid bacteria.
10. The use of a DNA sequence according to any of claims 1 to 3 or parts thereof for determining optimal fermentation and growth conditions of lactic acid bacteria.

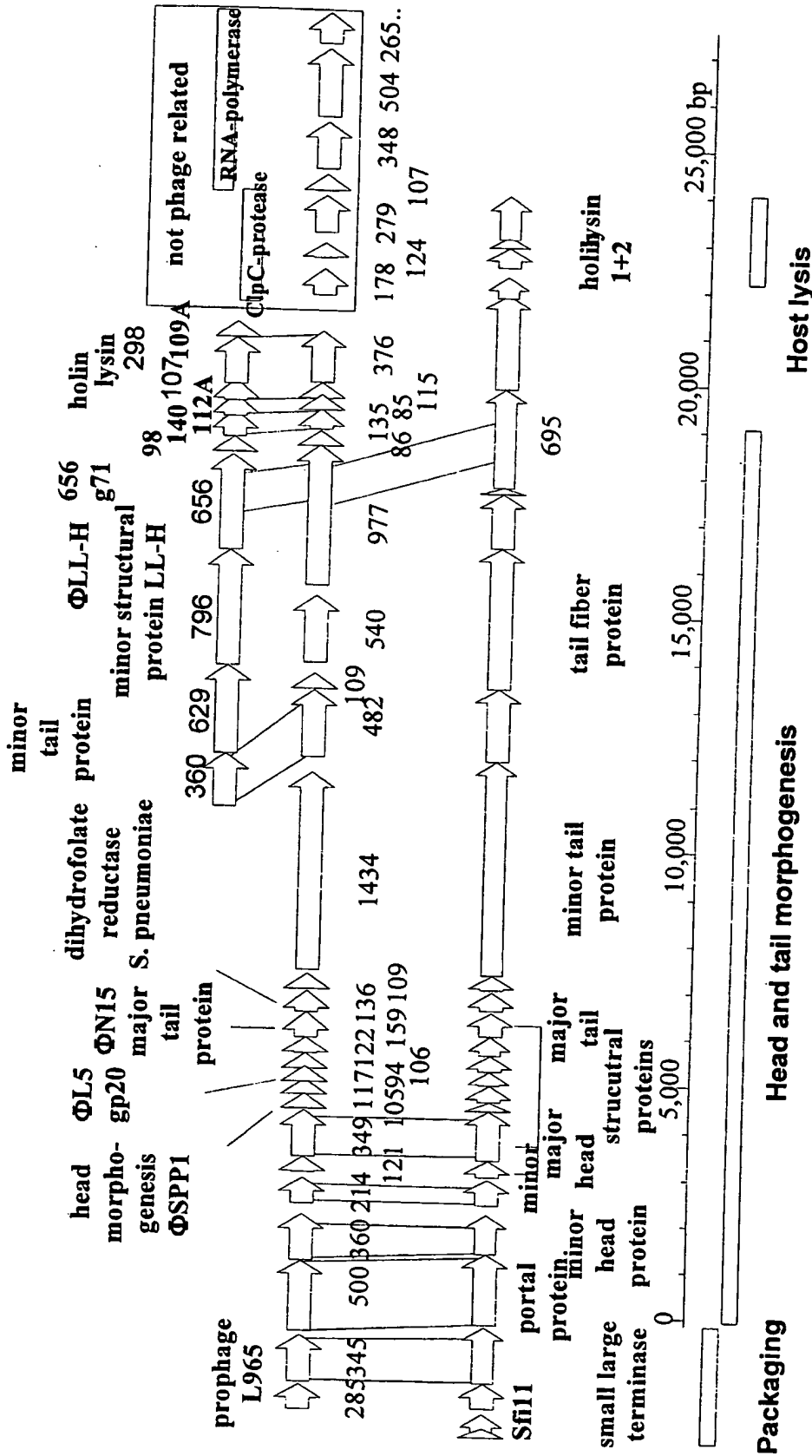


Fig. 1: Alignment of the gene map of L965 and the partial gene map of *Streptococcus thermophilus* bacteriophage Sfi11 and *Lactobacillus delbrueckii* bacteriophage LL-H.

2/3

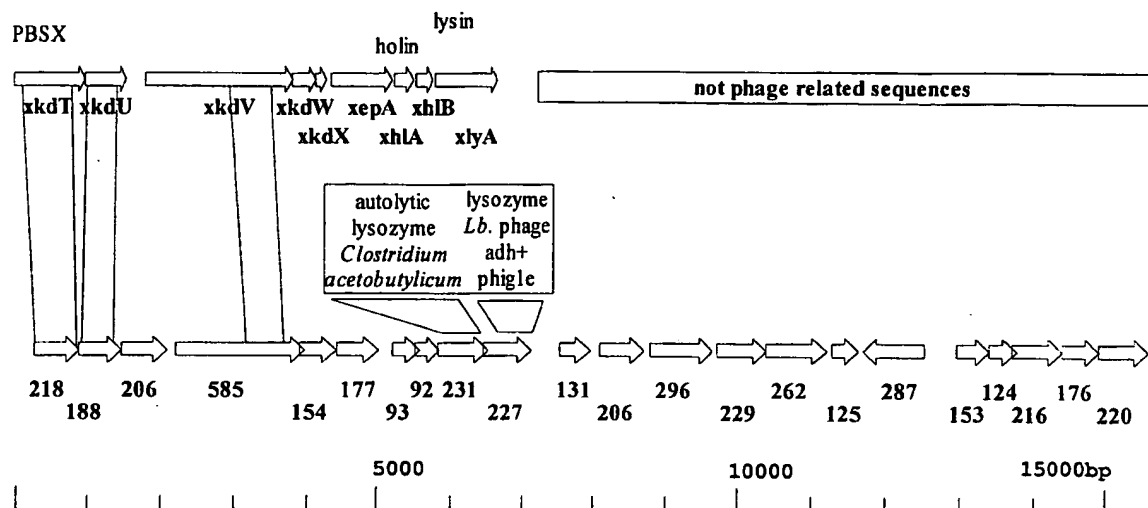


Fig. 2: Prediction of open reading frames in the contig L771.

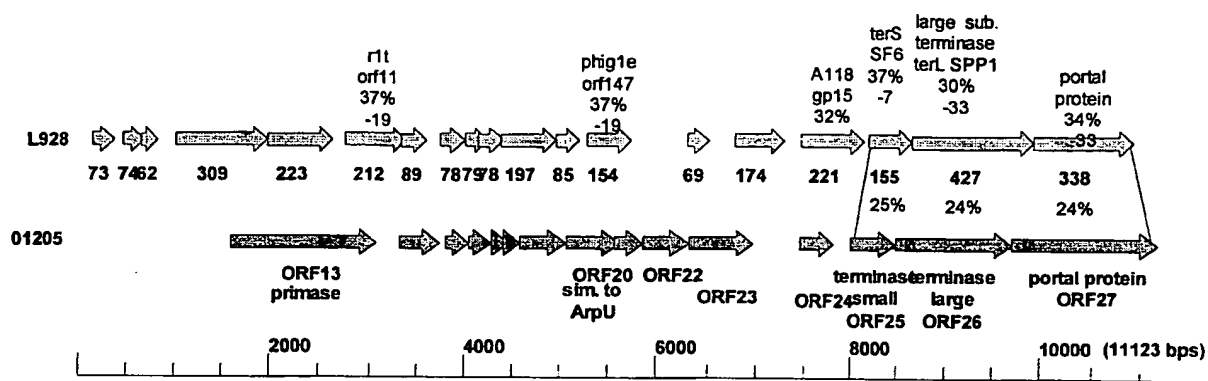


Fig 3: Contig L928 and predicted gene map with proposed functions.

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/06351

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, EPO-Internal, EMBL, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE EMBL 'Online! ID/AC: X13616, 19 March 1999 (1999-03-19) BARASH ET AL.: "ENTEROCOCCUS FAECALIS GENOME CONTIG. SEQ. ID NO: 679" XP002127572 abstract</p> <p style="text-align: center;">---</p>	1
X	<p>DATABASE EMBL 'Online! ID/AC: L03428, 5 October 1992 (1992-10-05) KURAMITSU: "STREPTOCOCCUS MUTANS G PROTEIN GENE SEQUENCE" XP002127573 abstract</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	2

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *O* document referring to an oral disclosure, use, exhibition or other means
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Date of the actual completion of the international search

12 December 2000

Date of mailing of the international search report

27/12/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Hagenmaier, S

INTERNATIONAL SEARCH REPORT

International Publication No

PCT/EP 00/06351

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! ID/AC: AF109874, June 1999 (1999-06) MCGRATH: "BACTERIOPHAGE TUC2009" XP002127574 abstract	3
A	--- KODAIRA ET AL.: "GENOME STRUCTURE OF THE LACTOBACILLUS TEMPERATE PHAGE PSI-gle: THE WHOLE GENOME SEQUENCE AND THE PUTATIVE PROMOTER/REPRESSOR SYSTEM" GENE, vol. 187, 1997, pages 45-53, XP002127566 cited in the application the whole document	
A	--- CHANDRY ET AL.: "ANALYSIS OF THE DNA SEQUENCE, GENE EXPRESSION, ORIGIN OF REPLICATION AND MODULAR STRUCTURE OF THE LACTOCOCCUS LACTIS LYTIC BACTERIOPHAGE SK1" MOL.MICROBIOL., vol. 26, no. 1, 1997, pages 49-64, XP000866448 cited in the application the whole document	
A	--- HENRICH ET AL.: "PRIMARY STRUCTURE AND FUNCTIONAL ANALYSIS OF THE LYSIS GENES OF LACTOBACILLUS GASSERI BACTERIOPHAGE PSI-ADH" J.BAC., vol. 177, no. 3, 1995, pages 723-732, XP002127567 the whole document	
A	--- VASALA ET AL.: "GENETIC AND BIOCHEMICAL CHARACTERIZATION OF THE LACTOBACILLUS DELBRUECKII SUSP. LACTIS BACTERIOPHAGE LL-H LYSIN" APPLIED AND ENVIROMENTAL MICROBIOLOGY, vol. 61, no. 11, 1995, pages 4004-4011, XP002127568 cited in the application the whole document --- -/--	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/06351

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LUCCHINI SACHA ET AL.: "The structural gene module in Streptococcus thermophilus bacteriophage variant phiSfill shows a hierarchy of relatedness to siphoviridae from a wide range of bacterial hosts." VIROLOGY, vol. 246, no. 1, 20 June 1998 (1998-06-20), pages 63-73, XP002154730 ISSN: 0042-6822 the whole document</p> <p style="text-align: center;">---</p>	
A	<p>KROGH ET AL.: "THE PHAGE-LIKE ELEMENT PBSX AND PART OF THE SKIN ELEMENT, WHICH ARE RESIDENT AT DIFFERENT LOCATIONS ON THE BACILLUS CHROMOSOME ARE HIGHLY HOMOLOGOUS" MICROBIOLOGY, vol. 142, 1996, pages 2031-2040, XP000857879 cited in the application the whole document</p> <p style="text-align: center;">---</p>	
A	<p>HENDRIX ET AL.: "EVOLUTIONARY RELATIONSHIPS AMONG DIVERSE BACTERIOPHAGES AND PROPHAGES: ALL THE WORLD'S A PHAGE" PNAS, vol. 96, March 1999 (1999-03), pages 2192-2197, XP002127570 the whole document</p> <p style="text-align: center;">---</p>	
A	<p>BRÜSSOW ET AL.: "MOLECULAR ECOLOGY AND EVOLUTION OF STREPTOCOCCUS THERMOPHILUS BACTERIOPHAGES-A REVIEW" VIRUS GENES, vol. 16, no. 1, 1998, pages 95-109, XP000867201 the whole document</p> <p style="text-align: center;">---</p>	
P,X	<p>DESIERE FRANK ET AL.: "Comparative genomics of the late gene cluster from Lactobacillus phages." VIROLOGY, vol. 275, no. 2, 30 September 2000 (2000-09-30), pages 294-305, XP002155242 ISSN: 0042-6822 the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-10

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	<p>ALTERMANN ET AL.: "PRIMARY STRUCTURE AND FEATURES OF THE GENOME OF THE LACTOBACILLUS GASSERI TEMPERATE BACTERIOPHAGE PSI-ADH" GENE, vol. 236, August 1999 (1999-08), pages 333-346, XP002127571 the whole document</p> <p>-----</p>	